Master Thesis

Design and Synthesis of Functional Polyamine Based on Novel Amino Glycidyl Ether

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Byeong-Su Kim

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Abstract

This thesis describes the design and synthesis of functional hyperbranched polyamines and their potential applications. We successfully synthesized hyperbranched polyglycerols containing amino functionality by using a novel Boc-protected amino ethanol glycidyl ether monomer (BAG). A series of hyperbranched Boc-protected polyamino glycerols (PBAG) were prepared through a one-pot anionic ring-opening multi-branching polymerization to yield PBAG with controlled molecular weights. Subsequent deprotection of PBAG yielded hyperbranched polyamino glycerols (PAG). ^{1}H, ^{13}C, and ^{15}N-NMR, GPC and MALDI-ToF measurements confirmed the successful polymerization of the hyperbranched PAG polymers. Because of its high biocompatibility, PAG is expected to be widely used in biological and biomedical fields. In addition, we have demonstrated that PAG can generate singlet oxygen species which can be used as photodynamic therapeutic agent. 9,10-anthracenediyl-bi(methylene)dimalonic acid (ABDA) assay and UV-Vis experiment were employed to confirm the generation of singlet oxygen species. We anticipate that PAG with high biocompatibility and singlet oxygen generation capability to be widely used in biomedical fields.

Contents

List of Figures

[part 1]

[part 2]

Figure 3. ^{1}H NMR spectra of (a) the BAG monomer measured in $CDCl_3$, (b) the $PBAG_{66}$ polymer, and (c) the deprotected PAG_{66} polymer (polymer 5) measured in D_2O. (d) Characterization Data for PAG Polymers Synthesized.

Figure 4. (a) Schematic representation of the potential side reaction of BAG monomer during the polymerization and its subsequent reaction with incoming new monomer. (b) A series of ^{1}H NMR spectra of Boc group deprotection for *tert*-butyl diethylcarbamate in $CDCl_3$ at different reaction time.

Figure 5. (a) Detailed ^{15}N-NMR spectrum of PAG_{66} (polymer 5) in DMSO-*d6*. (b) Expanded MALDI-ToF mass spectrum of the PAG_{13} (polymer 1) from 1600 to 2300 Da. The spacing of the signals corresponds to the mass of the respective monomers (AG: 161.2 g/mol).

Figure 6. DEPT spectra of PAG_{66} polymer (polymer 5) in DMSO-*d6* (b) Calculation of degree of branching based on the ^{13}C NMR spectra of PAG_{66} polymer (polymer 5).

Figure 7. (a) Detailed ^{13}C NMR spectrum of PAG_{66} polymer (polymer 5) in D_2O with assignment of respective linear, dendritic, and terminal groups within the structure. (b) In vitro cell viability assay of polymers. (Gray) PAG_{17} (polymer 2) and (white) PAG_{50} (polymer 4) determined by WST-1 assays using RAW264.7 cell lines.

Figure 8. (a) Potential singlet oxygen generation mechanism. (b) Molecular orbital diagrams for triplet oxygen, superoxide radical and singlet oxygen.

Figure 9. Absorbance decrease of 9,10-anthracenediyl-bi(methylene)dimalonic acid (ABDA) by singlet oxygen ($^{1}O_2$) production. Conditions: [PAG polymer] = 10 μM; [ABDA] = 100 μM; irradiation with 40% intensity of 1 sun light. Absorbance was obtained every 2.5 minutes.

List of Table

[part 2]

Part 1. Introduction of hyperbranched polyglycerols and its derivatives

1.1 Hyperbranched polymers

Hyperbranched polymer (HBP) is a type of synthetic macromolecules. With a tree-like structure.[1] Hyperbranched polymers have many terminal groups. Dendritic polymers are divided into fully branched polymers and hyperbranched polymers. The structure of the dendrimers and hyperbranched polymers are shown in Figure 1a.[2] The dendrimers have constant structure depending on the generation, but hyperbranched polymers have irregular structure because they have a randomly branched structure. The dendrimer must go through a lengthy and tedious multi-step process, but the hyperbranched polymers can be synthesized by one-step polymerization. Furthermore, hyperbranched polymers have many functional groups on the inner part as well as the outer part of the polymer. Because of these functional groups can be easily modified, they can be used as powerful tools for a variety of applications. Also, because there is no chain entanglement, processing ability is excellent. These properties make hyperbranched polymer very important in industry fields.

The parameter representing the hyperbranched structure is degree of branching (DB). Dendrimers have no linear structure and only branched structure and terminal structure. Therefore, DB of dendrimers is 1. On the other hand, DB of linear polymers is 0 because there is no terminal unit or branched unit. Since hyperbranched polymers have terminal unit, branched unit, and linear unit, the degree of branching of hyperbranched polymers is within the range of 0.4-0.6 (Figure 1b).[3]

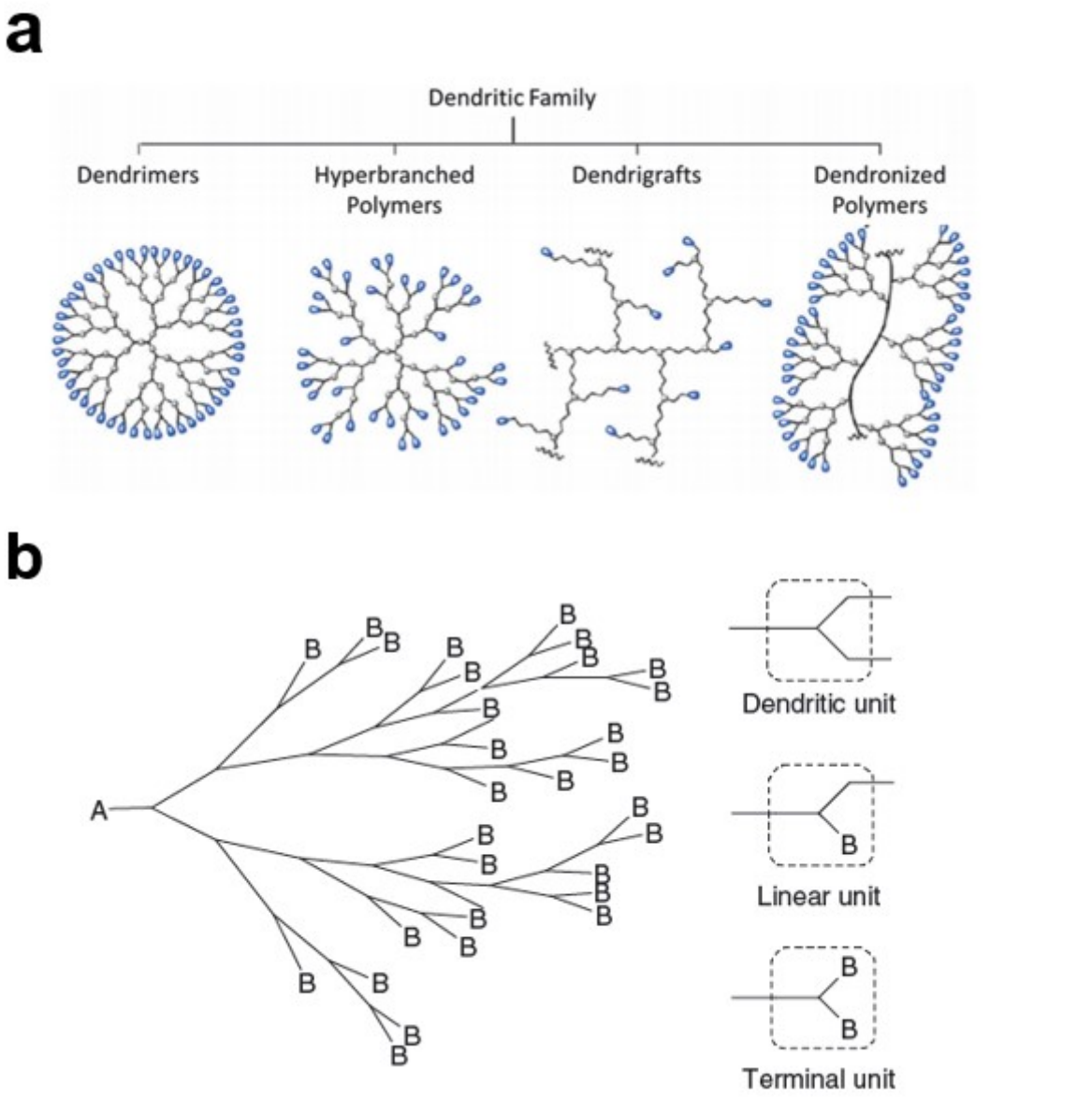

Figure 1. (a) Schematic illustration of the dendritic family. (b) Schematic illustration of hyperbranched polymer and structural unit of hyperbranched polymer from an AB_2 monomer. Reprinted with permission from *Chem. Soc. Rev.* **2015**, *44*, 4131–4144. Copyright 2015 Royal Society Chemistry.

$$DB = \frac{2D}{2D + \Sigma L}$$

The formula for calculating the degree of branching was created by Frey and co-workers.[4] This formula only applies to AB_m ($m \geq 2$) type monomers. In this formula, DB is determined by using NMR spectroscopy. The DB can be obtained by comparing the NMR integral values of linear unit, dendritic unit, terminal unit. Polymerization of AB_m monomer by slow monomer addition method gives well-controlled molecular weight and polydispersity.

Moore and co-workers first reported hyperbranched polymers with narrow PDI values by using slow monomer addition method. Since this discovery, many scientists have synthesized polymers with narrow polydispersity index by slow monomer addition method of AB_m monomer. Condensation polymerization was mainly used to polymerize AB_m monomer. However, due to the low molecular weight byproduct produced during the reaction, there was a limitation on the formation of high molecular weight polymers. On the other hand, the use of cyclic monomer eliminated the byproduct and made it possible to obtain high molecular weight easily. Frey and co-workers reported anionic ring-opening multi-branching polymerization of glycidol in 1999.[5] Glycidol is a cyclic molecule and AB_2 type monomer. Proton transfer of glycidol during polymerization results in hyperbranched structure as shown in Figure 2a.[5]

1.2 Polyglycerols

Poly(ethylene glycol)(PEG) is widely used in many fields such as medical, chemical, commercial, industrial and biomedical fields.[6,7] There are various reasons why PEG is used for variety purposes. First, PEG is non-toxic and soluble in water. This property makes PEG biocompatible. Therefore, PEG is used for cosmetics, biosensors, imaging, coating material and medicine. Second, PEG has a “stealth effect”. Conjugation of PEG with other drugs or proteins are called PEGylation.[8–10] “PEGylation” can increase the hydrodynamic radius of materials and improves the efficiency of biological product. However, PEG has some disadvantages and limitations. For example, (i) oligomers with a molecular weight of less than 400 exhibit cytotoxicity due to enzyme-catalyzed oxidation processes; (ii) PEG has a linear structure and only two hydroxyl end groups,

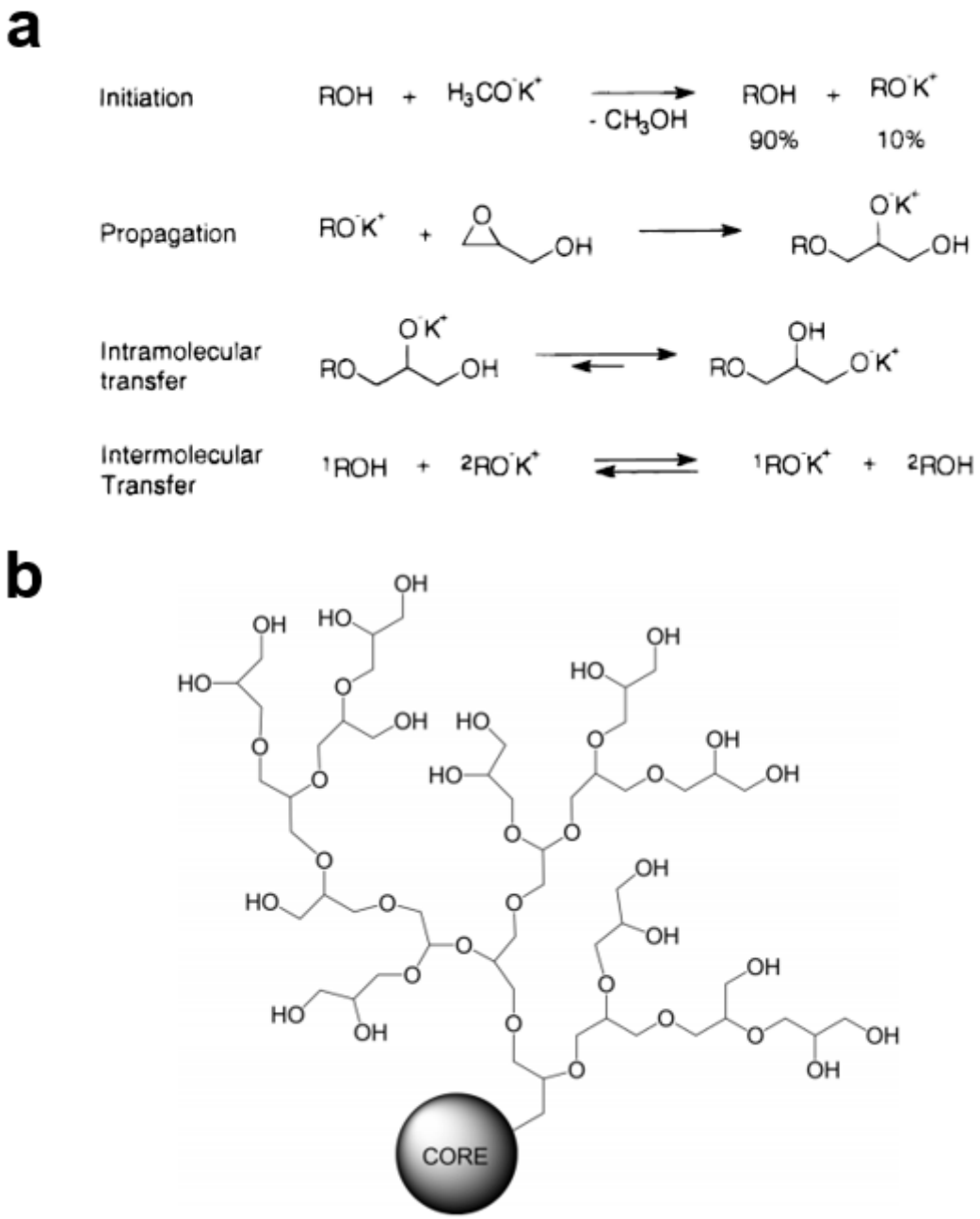

Figure 2. (a) Polymerization mechanism of glycidol. (b) structure of hyperbranched polyglycerol. Reprinted with permission from *Macromolecules* **1999**, *32*, 4240–4246. Copyright 1999 American Chemical Society.

which limits conjugation of drugs and proteins; (iii) synthesis of PEG is difficult due to the toxicity of ethylene oxide monomers and their gaseous form. To overcome this problem, PGs having a similar structure to PEG with multiple hydroxyl-functional groups can be an alternative to conventional PEG. The functionalized glycerol monomer could be synthesized in various architecture such as hyperbranched or linear structure as illustrated in Figure 3. Hyperbranched polyglycerols can be synthesized by anionic ring-opening multi-branching polymerization. The characteristics of hyperbranched polyglycerols have been widely studied.[11] By protecting the hydroxyl groups, branching of glycidol can be prevented. With this strategy, various polyglycerols with different architectures can be synthesized and defined from hyperbranched structure to complex structure (Figure 4). For example, the use of protected monomers such as ethoxyethyl glycidyl ether (EEGE) results in the linear polyglycerols (*lin*PG) that show a similar biocompatibility.[12] In fact, glycerol oligomers with a degree of polymerization of less than ten have been approved by the FDA for food and pharmaceutical additives and have been applied various fields for decades.

1.2.1 Functionalized polyglycerol

Many hydroxyl groups of PG provide reaction sites for modification and functionalization. The functionalized PG can be synthesized by initiation of functionalized molecules. The functionalized PG can make conjugation with other molecules. Because of this conjugation characteristics, PG has been widely used in various fields.[13] Core-functionalized polymers can be synthesized using a functionalized initiator. However, it requires the hydroxyl group containing initiator which is stable in the harsh and basic conditions during ring-opening polymerization. For this purpose, many initiators containing hydroxyl group, amino group, carboxylic acid group, aldehyde group and thiol moieties have been used to synthesize functional PG. Among these functional groups, amino group is attracting much attention because it is suitable for conjugation of biomaterials. In addition to amino groups, thiols have high affinity to metal surfaces and are used to fix the polymer on the substrate. For instance, Weinhart and co-workers reported shielded amine-functionalized initiator and thiol-functionalized initiator for anionic ring-opening polymerization. They used benzyl group to shield the functional

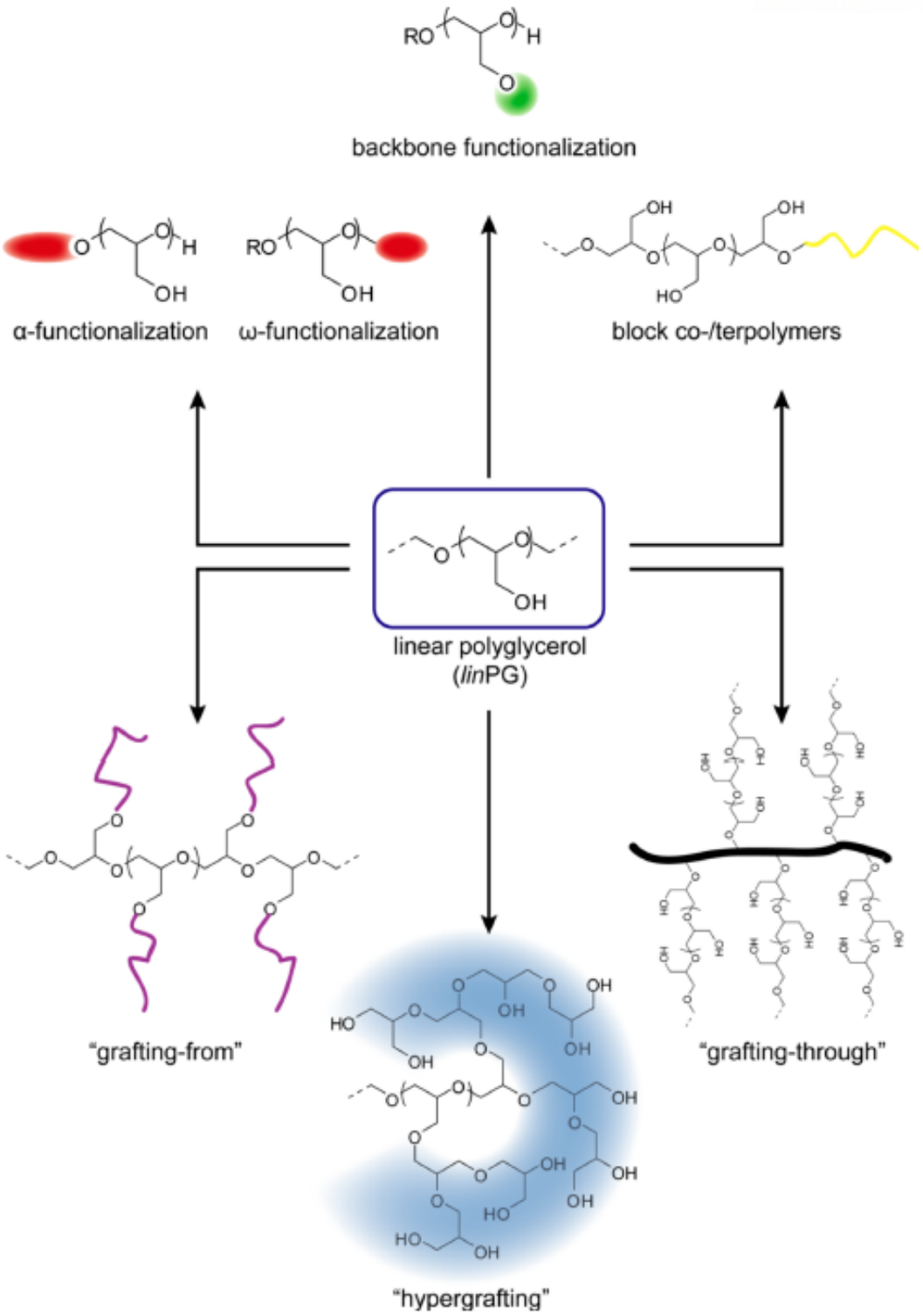

Figure 3. Various strategy to modify linear polyglycerols (*lin*PG). Reprinted with permission from *Biomacromolecules* **2014**, *15*, 1935–1954. Copyright 2014 American Chemical Society.

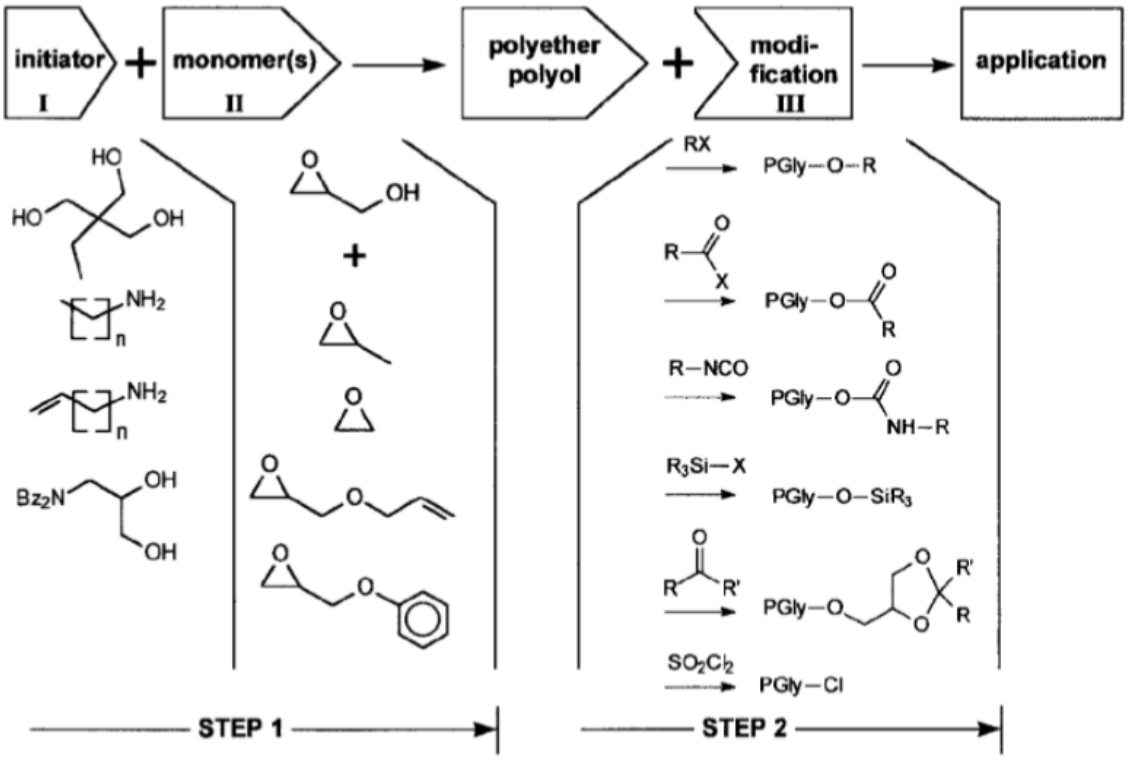

Figure 4. Overview of synthesis of functional polyether and polyols and its derivatives. Reprinted with permission from *Adv. Mater* **2000**, *12*, 235–239. Copyright 2000 John Wiley & Sons, Inc.

group to maintain stability during the polymerization. The deprotection of the protecting group gives us free amino and thiol-groups. Kim and co-workers reported synthesis of PG using a light-sensitive spiropyran as the initiator.[14] Hydrophobic spiropyran turns into a hydrophilic merocyannine when UV is applied. Therefore, the polarity of the PG will be changed and afford micelles which is sensitive to light. The epoxide monomers containing functional groups such as triple bonds, double bonds and hydroxyl group are able to synthesize functionalized PG. Especially, glycidol and epichlorohydrin have been widely used as building blocks. Functionalized monomers are synthesized by nucleophilic attack of functional group such as amine and hydroxyl group in many steps. The copolymerization of glycidol and the functionalized epoxide monomer is successfully carried out by anionic ring-opening polymerization.

Interest in biodegradable polymers is increasing day by day. Therefore, many scientists researched that how to introduce degradable cross linker into the epoxide monomer. Shenoi *et al*. synthesized various functionalized epoxide monomer with ketal linkage by using anionic ring-opening polymerization.[15] Synthesized polymers are degraded by pH and temperatures. Specially, the degradation by the acidic condition could be fine-tuned by changing the ketal structure of the monomer. Kim and co-workers reported functional glycerol monomer containing disulfide bond.[16] Disulfide glycerol monomer (SSG) was homopolymerized and copolymerized with glycidol by anionic ring-opening polymerization. Because disulfide bond is degraded under redox environment, the resulting polymer has property of degradation under reduction-oxidation environment. In another approach, modification of pendant hydroxyl groups of PG has been studied. The pendant hydroxyl group can be changed into many functional groups such as carboxylic acid group, amine group and carbonyl group. The simplest modification is methylation of pendant hydroxyl group. Hoffman and co-workers firstly reported methylation of PG.[17] Interestingly, polarity of the PG was completely changed by methylation. In addition, esterification of hydroxyl group of PG and aliphatic acryl chlorides also reported.[18] They synthesized polymers in linear and hyperbranched structures.

1.2.2 Biomedical application

PG is attracting significant attention as a unique biocompatible polymer. PG is biocompatible, due to the structure similarity to PEG which is widely used in biochemistry, biomedical and industrial fields.[19] Brooks and co-workers reported biocompatibility of linear PG, hyperbranched PG and PEG in 2006.[12] All polymers showed high biocompatibility in vivo and in vitro assays. Figure 5 shows the high biocompatibility of PG. As a result of these experiments, high molecular weight PG and their derivatives have been used for biochemistry,[20,21] conjugation chemistry[22] and drug delivery.[23,24] Many scientists noted biocompatible polymer as protein or drug delivery carriers.[25] The combinations of anticancer drug and biocompatible polymer result in increased drug accumulation in cancer cell. The increase in efficiency of these drugs is called enhanced permeability and retention effect (EPR). With this conjugation, the drug can penetrate better into the tumor.[26,27] The hyperbranched PGs is a promising material for drug delivery due to their hydroxyl functional groups which make them easily modified for biomedical application.[28,29] Kim and co-workers have conjugated hyperbranced polyglyerols with doxorubicin, chemotherapeutic agent, and increased efficiency in 2012. Haag and co-workers reported novel photo-responsive polycation which have a star-like amine shell and biocompatible hyperbranched polyglycerols core.[30,31] These polymers have positive charges at specific pH with the polyether core. Therefore, they have biocompatibility and good gene transfection efficiency.

1.3 Polyamines

Amines are widely used in the chemical industry because of their nucleophilic nature. Amines are used in variety of applications such as food, detergents, lubricants and agricultural chemicals.[32,33] Amines are attracting attention as a material for composite and polymers. Therefore, amines are important monomer to synthesize polyamides, polyureas, polyurethanes which are widely used in automobile, healthcare and architecture applications. In particular, the production of polyamides is expected to increase by 10% annually. In addition the development of polyhydroxyurethanes has increased the demand for amines as hardeners of polycyclic carbonates. Amines are mainly available in the form

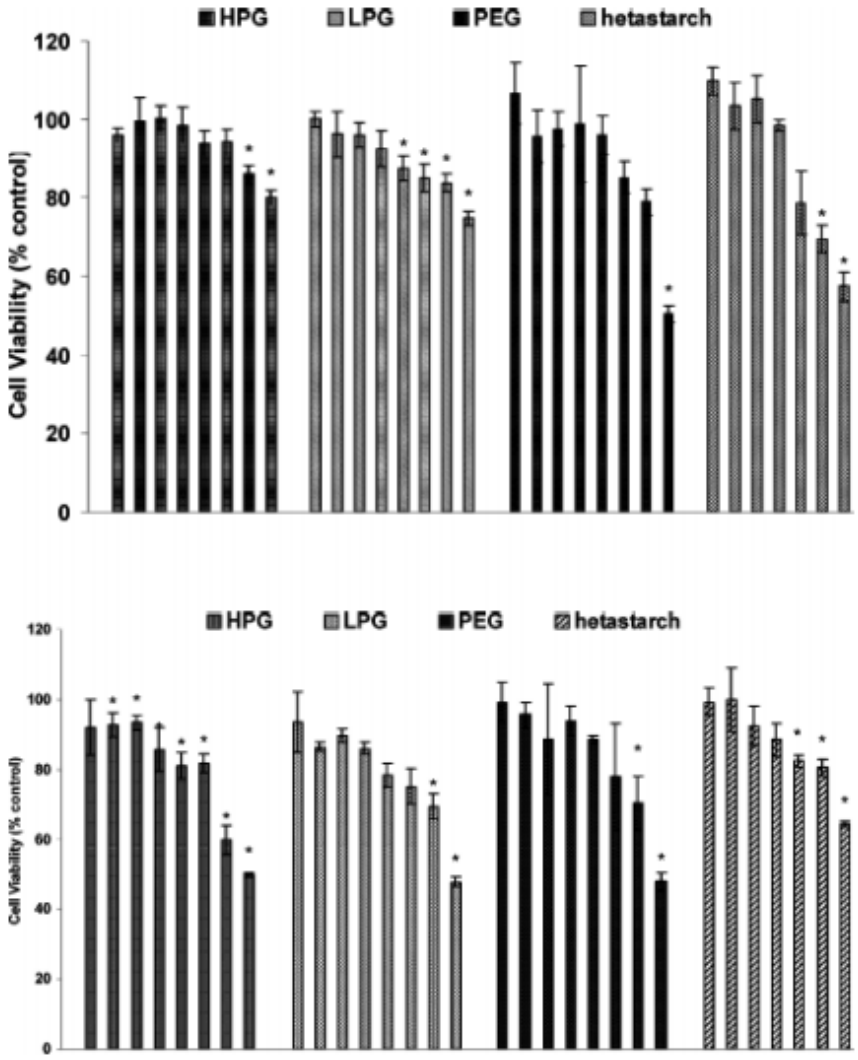

Figure 5. Cytotoxicity of hyperbranched polyglycerols (HPG), linear polyglycerols, Poly(ethylene glycol) and hetastarch. The top panel was studied under L-929 cell conditions and the bottom panel was studied under human umbilical vein endothelial cells (HUVEC) conditions. Reprinted with permission from *Biomacromolecules* **2006**, *7*, 703–709. Copyright 2006 American Chemical Society.

of amides or salts to avoid carbonates by carbon dioxide. Ammonia plays an important role in the synthesis of amines and many commercial amines are synthesized from ammonia. However, amination of alcohol is also possible to synthesize amine. This reaction is very easy to purify because water is generated as byproduct. Despite the increased interest in bio-based monomers and polymers, especially polyamides, the number of natural amines available is extremely small. Academia and industry are looking for novel amine containing monomers and polymers.

1.3.1 Polyamine functions

Polyamines play many roles in a wide variety of organisms.[34-37] For example, in mammals, polyamines function in diverse physiological processes including immunity, aging, hair growth and wound healing. Accordingly, the cellular concentrations of polyamines reflect these functions and vary widely according to cell type and context. In terms of cellular mechanisms, polyamines play important roles in messenger RNA (mRNA) translation and stability, both in a global sense as well as in specific cases. In addition, they are reported to modulate kinase activities, small RNA methylation, transcriptional regulation, microtubule assembly and ion channel regulation. Polyamines that are widely used in the biomedical field are shown in Figure 6.

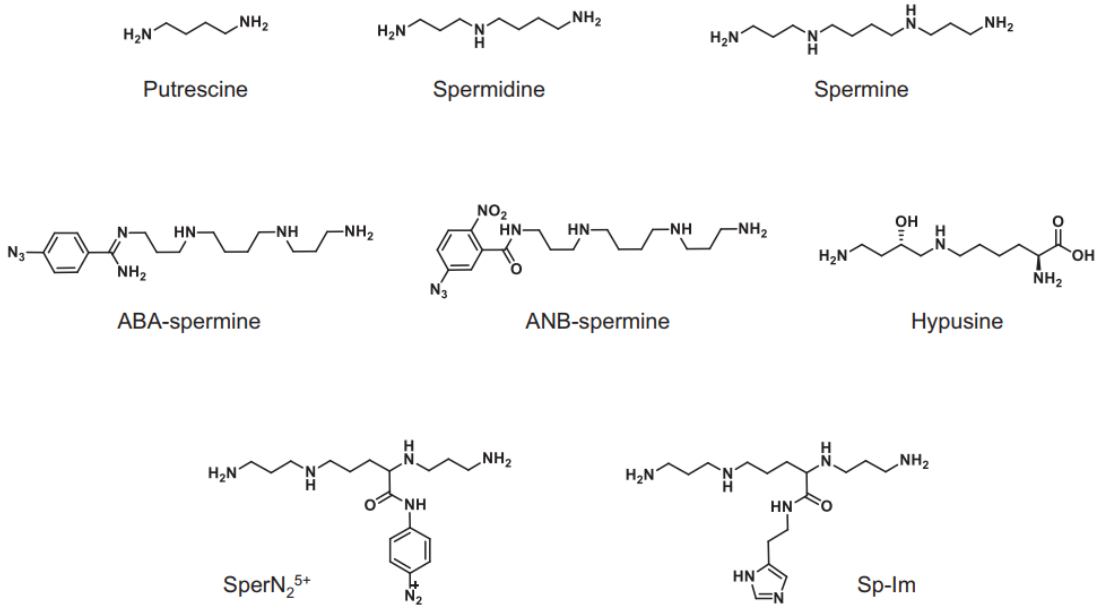

Figure 6. Chemical structure of the polyamines and polyamine analogues. Adapted with permission from *Nucleic Acid Res.* **2014**, *42*, 11275–11290. Copyright 2014 Oxford University Press.

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Part 2. Hyperbranched polyamines based on novel amino glycidyl ether

1. Introduction

Polyamines attracted significant attention owing to their wide range of functions in industry and biotechnology.[1–3] Polymers containing amine groups such as poly(ethylene imine) and poly(propylene imine) are promising CO_2 absorbent due to their high amine content.[4,5] Polyamines are also effective chelating agents used to dissolve metal ions in organic solvents and used as a hardener with epoxy resin.

In nature, polyamines are essential molecules supporting the structure, conformation, and function of many key biological molecules including nucleic acids and proteins. Naturally occurring polyamines such as spermine and spermidine are involved in cell growth, maintenance of membrane stability, regulation of programmed cell death and free radical scavenging.[6,7] Additionally, with of their cationic properties under physiological conditions, polyamines possess high potential as a vector for gene therapy. As a notable example, poly(ethylene imine) is commonly used as a gene transfer vector with a high transfection efficiency due to the proton sponge effect;[8,9] however, this high charge density often leads to significant toxicity to cells.[10,11] Thus, various methods have been proposed to lower the cytotoxicity of cationic polyamines.[12] For example, poly(ethylene glycol) (PEG) has often been conjugated to reduce its toxicity by shielding the positive charges.[13,14]

As an alternate approach, novel amine-containing monomers are introduced to synthesize polyamines with a biocompatible polyether backbone. For example, Frey and co-workers have reported various amine containing monomers such as *N,N*-dibenzyl amino glycidol,[15] *N,N*-diallyl glycidyl amine,[16] *N,N*-diethyl glycidyl amine,[17] and epicyanohydrin.[18] Moller and co-workers also reported poly(glycidol-*co*-glycidyl amine), and poly(glycidol)-*b*-poly(glycidyl amine).[19] In another worthwhile effort, Satoh and co-workers have reported the preparation of polyethers with various pendant amine groups using *N,N*-disubstituted glycidyl amine derivatives.[20] Kim group has also recently reported the use of protected butanolamine glycidyl ether for copolymerization with glycidol to enhance the biocompatibility of the resulting polyamines.[21] Many of the polymers developed thus far retained the primary amine groups and polymerized via the glycidyl amine monomer, leading to the synthesis of linear polyamine with a polyether backbone. In contrast to these previous reports, here we focused on the synthesis of hyperbranched polyamines with a protected monomer approach.

In continuation of our endeavor to develop functional hyperbranched polyethers for biomedical applications, herein we report the one-pot synthesis of hyperbranched polyglycerols possessing amino functionality by using a Boc-protected aminoethanol glycidyl ether monomer (BAG; Figure 1). Specifically, t-butyl (2-hydroxyethyl)(2-(oxiran-2-ylmethoxy) ethylcarbamate was designed and polymerized through an anionic ring-opening multibranching polymerization to yield a series of hyperbranched PBAG with controlled molecular weights and relatively low molecular weight distributions. Subsequent deprotection of PBAG yielded amino-containing hyperbranched polyamino glycerols (PAG). We also demonstrated the superior biocompatibility of the prepared PAG via a cell viability assay.

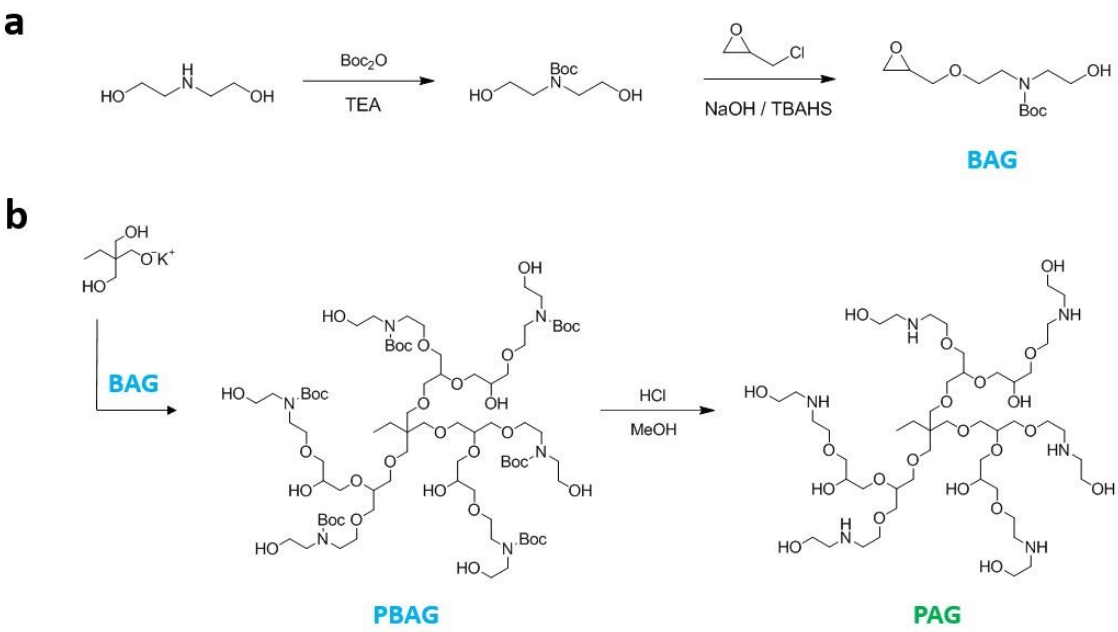


Figure 1. Synthetic scheme of (a) the BAG monomer and (b) the anionic ring-opening polymerization of PBAG and subsequent deprotection to yield PAG.

2. Experimental Section

2.1 Materials

All reagents and solvents were purchased from Sigma-Aldrich and Acros unless otherwise stated. Deuterated NMR solvents such as $CDCl_3$ and D_2O were purchased from Sigma-Aldrich.

2.2 Analysis method

^{1}H- and ^{13}C-NMR spectra were measured using a 400-MR DD2 (400 MHz) spectrometer with $CDCl_3$, D_2O and DMSO-d_6 as solvents, and chemical shifts were recorded in ppm units with TMS as an internal standard. The weight-averaged (M_w) molecular weights and molecular-weight distribution (M_w/M_n) were measured using gel permeation chromatography (GPC, Agilent Technologies 1200 series) with a poly(methyl methacrylate) (PMMA) standard and dimethylformamide (DMF) as an eluent at 30 °C with a flow rate of 1.00 mL/min. ^{15}N NMR spectra was measured using a Varian VNMRS 600 MHz NMR spectrometer with DMSO as solvents and formamide as a standard. Matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-ToF) measurements were carried out on an Ultraflex III MALDI mass spectrometer. α-cyano-4-hydroxycinnamic acid was used as the matrix. A 10 g/L solution of the polymer in acetonitrile and 10 g/L solution of the matrix solution were prepared separately. A 1.0 μL aliquot of the mixture was applied to a target plate, and the solvent was evaporated before measurement. Differential scanning calorimetry (DSC) was carried out using a DSC (Q200 model, TA Instruments) in the temperature range from −80 to 20 °C at a heating rate of 10 K/min under nitrogen. The zeta potential was measured using a Malvern Zetasizer Nano-ZS (ZEN3600, Malvern, UK).

2.3 Reaction to protect diethanolamine

The precursor, *t*-butyl bis(2-hydroxyethyl)carbamate was synthesized similar to the literature protocol with slight modifications.[22] A solution of di-*tert*-butyl-dicarbonate (22.9 mL, 99.9 mmol) in CH_2Cl_2 (50 mL) was added to a solution of diethanolamine (10 g, 95.1 mmol) and triethylamine (13.9 mL, 99.9 mmol) in CH_2Cl_2 (30 mL) dropwise over 1 h using a dropping funnel at room temperature. The mixture was stirred at room

temperature for 6 h, diluted with CH_2Cl_2, and extracted with water and brine. The organic phase was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash column chromatography with 10% hexane in ethyl acetate as the eluent to obtain a pure compound as pale-yellow oil (13.67 g, 70%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 3.85 (d, 6H, J = 55.9 Hz), 3.42 (s, 4H), 1.46 (s, 9H).

2.4 Reaction for synthesizing BAG

An aqueous solution of sodium hydroxide (3.90 g, 50 wt%), epichlorohydrin (10.7 g, 116 mmol) and tetrabutylammonium hydrogen sulfate (TBAHS, 1.65 g, 4.87 mmol) was stirred at 0 °C. Then, a solution of t-butyl bis(2-hydroxyethyl)carbamate (10 g, 48.7 mmol) in THF (30 mL) was slowly added dropwise over 30 min and stirred at room temperature for additional 15 h.[23] To this reaction mixture, CH_2Cl_2 was added to extract the product and washed with water and brine to neutrality. The organic layer was dried with anhydrous sodium sulfate and concentrated under reduced pressure. The residue was purified by flash column chromatography with 17% hexane in ethyl acetate to give the BAG monomer as a pale-yellow viscous liquid (4.1 g, 32%). The synthesis of the BAG monomer was successfully identified via different spectroscopic and mass analyses, including ^{1}H and ^{13}C NMR (Figure 2) and ESI-MS. ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 3.88-3.21 (m, 11H), 3.21-3.06 (m, 1H), 2.88-2.73 (m, 1H), 2.62 (s, 1H), 1.47 (s, 9H). ^{13}C NMR (101 MHz, CDCl3): δ ppm 156.03, 80.06, 77.11, 71.69, 70.34, 62.20, 52.21, 50.55, 48.97, 43.92, 28.32. MS (m/z+, Na+, ESI+) calcd for $C_{12}H_{23}NO_2$ 284.3, found 283.9.

2.5 Synthesis of PBAG (Polymer 2)

1,1,1-Trimethylolpropane (TMP) (26.8 mg, 0.2 mmol) was placed in a one-neck round bottom flask. Potassium methoxide in methanol (25.0 wt %, 22.4 μL, 0.0758 mmol) was diluted with 0.70 mL of methanol and then added to the flask and stirred for 30 min at room temperature under an argon atmosphere. Methanol was removed with high vacuum for 4 h at 60 °C to yield a white salt, the initiator. The flask was purged with argon and heated to 90 °C. A t-butyl (2-hydroxylethyl)(2-oxiran-2-ylmethoxyl) ethyl carbamate (BAG) (1.0 g, 19.1 mmol) monomer was added slowly over 12 h via a syringe pump.

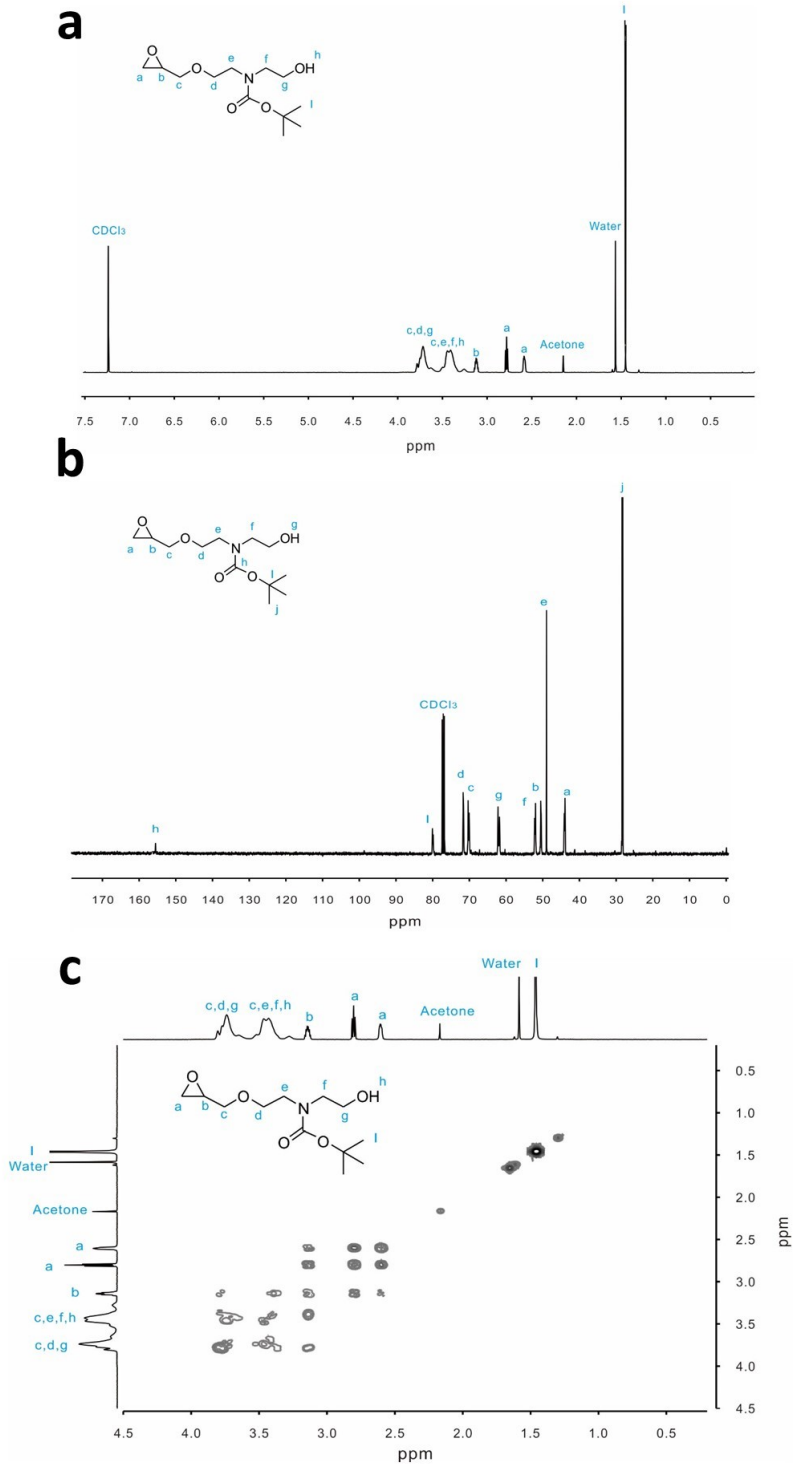


Figure 2. ^{1}H NMR spectrum of BAG monomer in $CDCl_3$. (b) ^{13}C NMR spectrum of BAG monomer in $CDCl_3$. (c) COSY spectrum of BAG monomer in D_2O.

After complete addition of the monomer, the solution was stirred for additional 36 h. The resulting homopolymer was quenched by adding 1.0 mL of methanol; the polymer solution was then precipitated into cold diethyl ether, and the precipitate was washed twice using diethyl ether. The resulting $PBAG_{17}$ polymer was dried under vacuum at 60 °C for 1 day. The M_n of $PBAG_{17}$ (polymer 2) was 4524 g/mol, as calculated from the NMR data using the following equation: number of repeating units (BAG) = 218.39 (integration value of polyether backbone) / 13 (number of protons of polyether backbone) = 16.80; M_n = 261.32 (molecular weight of the BAG monomer) × 16.80 + 134.17 (molecular weight of TMP) = 4524.35 g/mol. There are some errors in the molecular weight measurement using NMR measurement, we used the 4500 g/mol as a M_n value determined from the NMR. Typical monomer conversion was determined to be between 87–95% for all polymers synthesized with isolated yields of around 85% after purification in ether.

2.6 Process of removing the Boc protecting group

The Boc-protected polyamino glycerols (PBAG) polymer (polymer 2) was dissolved in CH_2Cl_2 with 1.0 mL of 1.0 M HCl and stirred at room temperature for 2 h. The reaction mixture was removed under reduced pressure and the resulting deprotected polymer was dissolved in 1.0 mL of methanol; the homogeneous polymer solution was then precipitated into excess diethyl ether, and the precipitate was washed twice using diethyl ether. The resulting deprotected PAG_{17} polymer was dried under vacuum at 60 °C for 1 day, which gave a pale-yellow viscous liquid (yield 97%).

2.7 Cell viability Assay

Murine macrophage cell line, RAW264.7, was purchased from the Korean Cell Line Bank (Seoul, Korea). Cytotoxicity assays were performed using the traditional WST-1 assay. Cells were seeded in 96-well plates at a density of 1 × 105 cells per well and incubated for 24 h in 5% CO2 at 37 °C. RAW264.7 cells were cultured with Roswell Park Memorial Institute medium (RPMI; WELGENE) with 10% fetal bovine serum (FBS) and 1% penicillin−streptomycin. Each well was then treated with various concentrations of PAG solutions (polymer 2 and 4) and incubated for an additional 24 h. For the WST-1 assays, each well was filled with 10 μL of EZ-Cytox (EZ-3000; Dogen bio). After

incubation for 1 h, the plates were gently shaken for 1 min before the absorbance was measured. The absorbance of the solution was recorded at a wavelength of 450 nm using 600 – 650 nm as the reference.

2.8 Reactive Oxygen Species Assay : 9,10-anthracenediyl-bi(methylene)dimalonic acid (ABDA) assay

A 100 mM ABDA solution in DMSO was prepared. Then we added the PAG polymer solution 10 mM into the ABDA solution. Total concentration ratio of PAG and ABDA becomes 1:10 (10 mM : 100 mM). We used solar simulator (IQE-200, Newport Co.) to light the solutions. After light exposure, UV-Vis is used to measure the degradation of ABDA.

3.Results and Discussion

3.1 Characterization of hyperbranched polyamines

The BAG monomer and the PAG polymer were synthesized by the methods described in Figure 1. As a first step, diethanolamine was protected with di-*t*-butyl dicarbonate (Boc_2O) and triethylamine (TEA) in CH_2Cl_2 to shield amine groups which have a high reactivity. The synthesized *t*-butyl bis(2-hydroxyethyl)carbamate was reacted with epichlorohydrin to yield a Boc-protected aminoethanol glycidol monomer (BAG). A variety of spectroscopic and mass spectrometric analyze confirmed successful synthesis of the BAG monomer. (see Figure 2 and Figure 3).

After the BAG monomer was prepared, we used the anionic ring-opening multibranching polymerization using a potassium alkoxide initiator that was synthesized via the reaction of potassium methoxide solution and trimethylolpropane (TMP). As described in previous studies,[24] we performed a slow monomer addition of BAG monomer to the deprotonated TMP initiator and polymerized at 90 °C for 48 h to synthesize the polymers in a controlled manner. The successful synthesis of PBAG polymers was confirmed by ^{1}H NMR and GPC measurements (Figure 3). As shown in Figure 3, The characteristic proton peak of BAG monomer and PAG polymer was confirmed by ^{1}H NMR. Furthermore, the number average molecular weight was calculated by comparing the peak integrals of methylene groups of the TMP initiator (peaks at 0.75 and 1.25 ppm, respectively) and polyether backbone (peaks at 3.0–4.0 ppm). The PAG was easily obtained by reacting PBAG with hydrochloric acid for 2 h. Deprotection of the Boc group of PBAG could be easily confirmed by disappearance of the peak of the string t-butyl groups at 1.34 ppm in the ^{1}H NMR (Figure 3c). The synthesized PBAG and deprotected PAG polymers were highly soluble in many solvents such as chloroform, diethyl ether, and tetrahydrofuran.

Surprisingly, PBAG polymer has a broad backbone peak (3.0-4.0 ppm), but the PAG has a sharp back bone peak (3.0-4.0 ppm). We assumed that the aqueous solubility of PAG increased when the bulky hydrophobic Boc protecting groups were romoved.[25] The synthesized PBAG polymers were further analyzed using GPC analysis. PBAG was analyzed instead of PAG because the secondary amine group of PAG polymer interacted

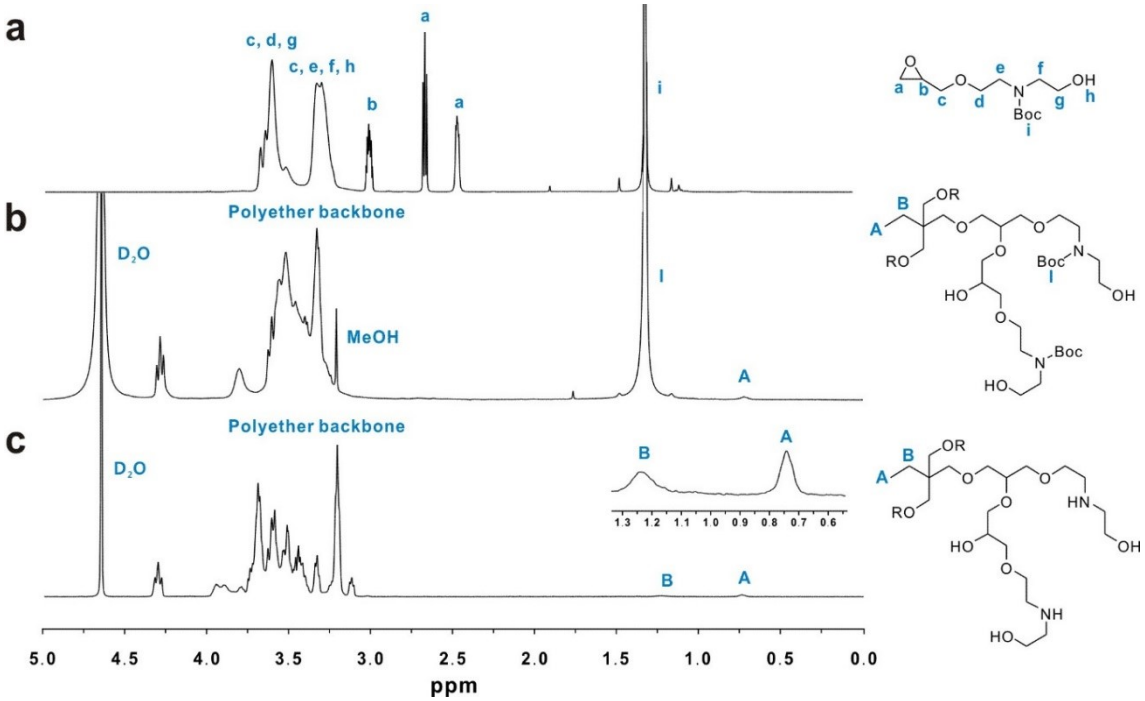


Figure 3. ^{1}H NMR spectra of (a) the BAG monomer measured in $CDCl_3$, (b) the $PBAG_{66}$ polymer, and (c) the deprotected PAG_{66} polymer (polymer 5) measured in D_2O.

Table 1. Characterization data for PAG polymers synthesized

No	Polymer composition (NMR)[a]	M_n (NMR)		M_w[b]	M_w/M_n[b]	T_g / °C (DSC)		Zeta potential / mV
		PBAG	PAG	PBAG		PBAG	PAG	
1	PAG_{13}	3500	2300	3400	1.19	-28.4	-33.5	14.1±1.0
2	PAG_{17}	4500	2800	4100	1.25	-15.4	-37.5	16.7±0.7
3	PAG_{33}	8700	5300	8200	1.39	-14.0	-37.5	19.1±2.2
4	PAG_{50}	13300	7700	9400	1.67	-21.0	-31.2	17.0±0.9
5	PAG_{66}	17400	10800	10600	1.83	-25.6	-43.8	23.7±1.4

[a] Composition is determined via ^{1}H NMR spectroscopy. [b] Measured using GPC-RI in DMF with a PMMA standard.

with the solid particles in the GPC column. The GPC data showed a wide range of molecular weight and monomodal distribution (Table 1). It meant that polymers were free from byproducts. Especially, the weight average molecular weight of the PBAG polymers was found to be 3400–10600 g/mol with a polydispersity index (M_w/M_n) of 1.19–1.83 determined by GPC using PMMA as a standard because of the existence of the hydrophobic Boc protecting group. Normally, the molecular weights of polymers obtained by GPC measurements is similar to the molecular weight obtained by ^{1}H NMR; however, in the case of the high molecular weight polymers $PBAG_{50}$ and $PBAG_{66}$, there was a difference in measured molecular weight. As longer reaction times are required to synthesize high molecular weight polymers, the harsh reaction conditions (strong base and high temperature) can deprotect the Boc group during polymerization. As a result, the secondary amine group might be involved as a reactive group during the polymerization, leading to a denser structure (Figure 4a). A similar phenomenon was observed in our previous report of the Boc-protected butanolamine glycidyl ether system.[16]

To confirm the existence of side reactions, we conducted a model experiment using a model compound, *tert*-butyl diethylcarbamate (Figure 4b). Under identical reaction condition, approximately 5% of the Boc group was deprotected, revealing a potential side reaction during the polymerization.

Moreover, we could identify the presence of tertiary amine group in the polymeric backbone resulting from the potential side reaction of the deprotected Boc group during the polymerization by employing ^{15}N NMR (Figure 5a). As similarly determined in the structure of branched poly(ethylene imine) (PEI) in distinguishing between the secondary and tertiary amine groups,[26] we could monitor the side reaction during the polymerization. However, it should be noted that the fraction of the tertiary amine group is significantly lower than that of secondary amine groups in line with the model reaction conducted.

MALDI-ToF spectrometry was performed to identify the insertion of the TMP initiator and the functional monomer segment in the PAG polymers. As shown in Figure 5b, the spacing of the signals corresponds to the mass of the respective monomers in the PAG polymer, which are present to varying degrees, unambiguously demonstrating the successful polymerization of PAG. For example, the mass peak at 1946.56 m/z corresponded to the polymer with TMP as an initiator, 11units of monomer, and K^+ as a

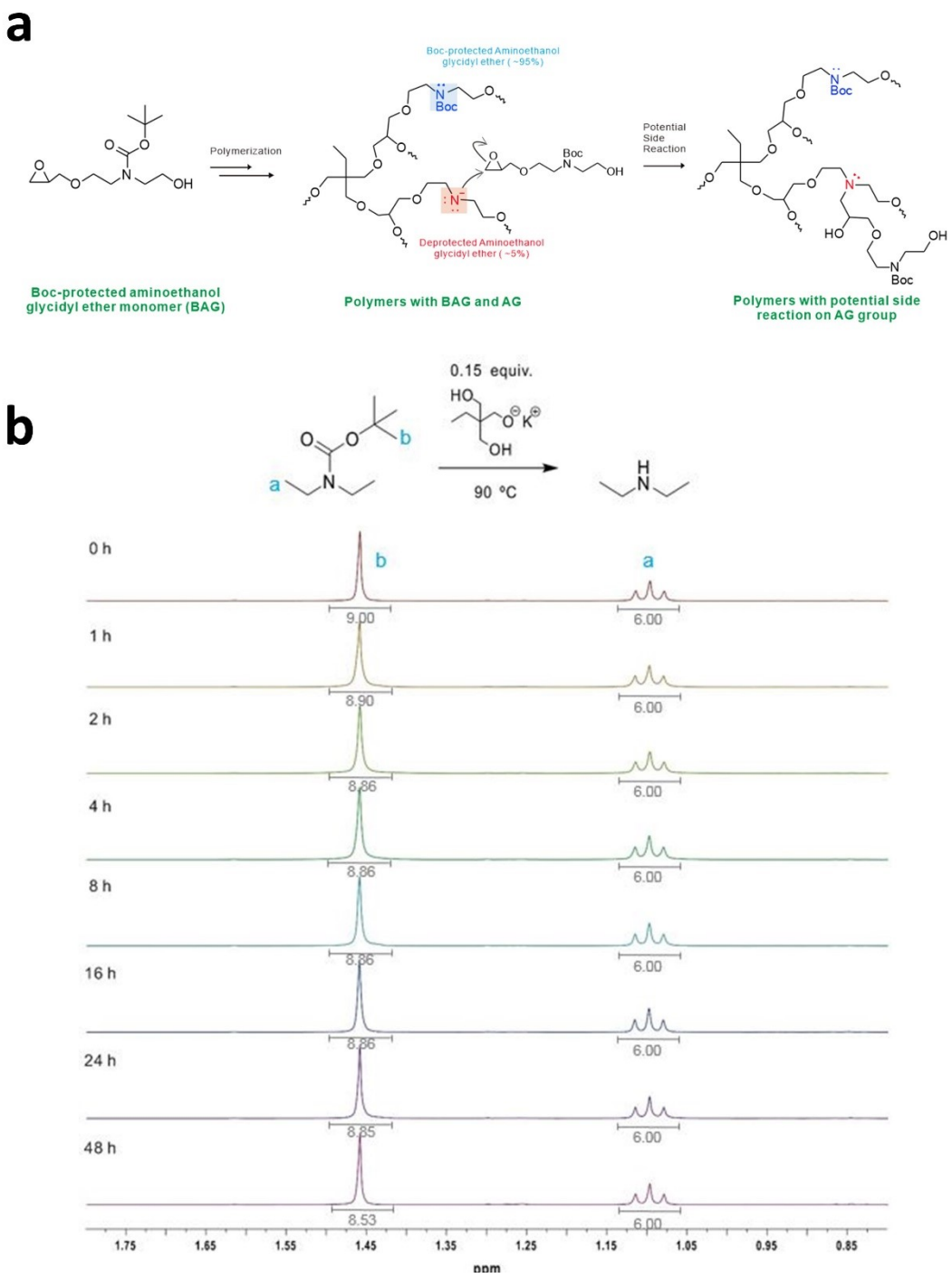


Figure 4. (a) Schematic representation of the potential side reaction of BAG monomer during the polymerization and its subsequent reaction with incoming new monomer. (b) A series of ^{1}H NMR spectra of Boc group deprotection for *tert*-butyl diethylcarbamate in $CDCl_3$ at different reaction time.

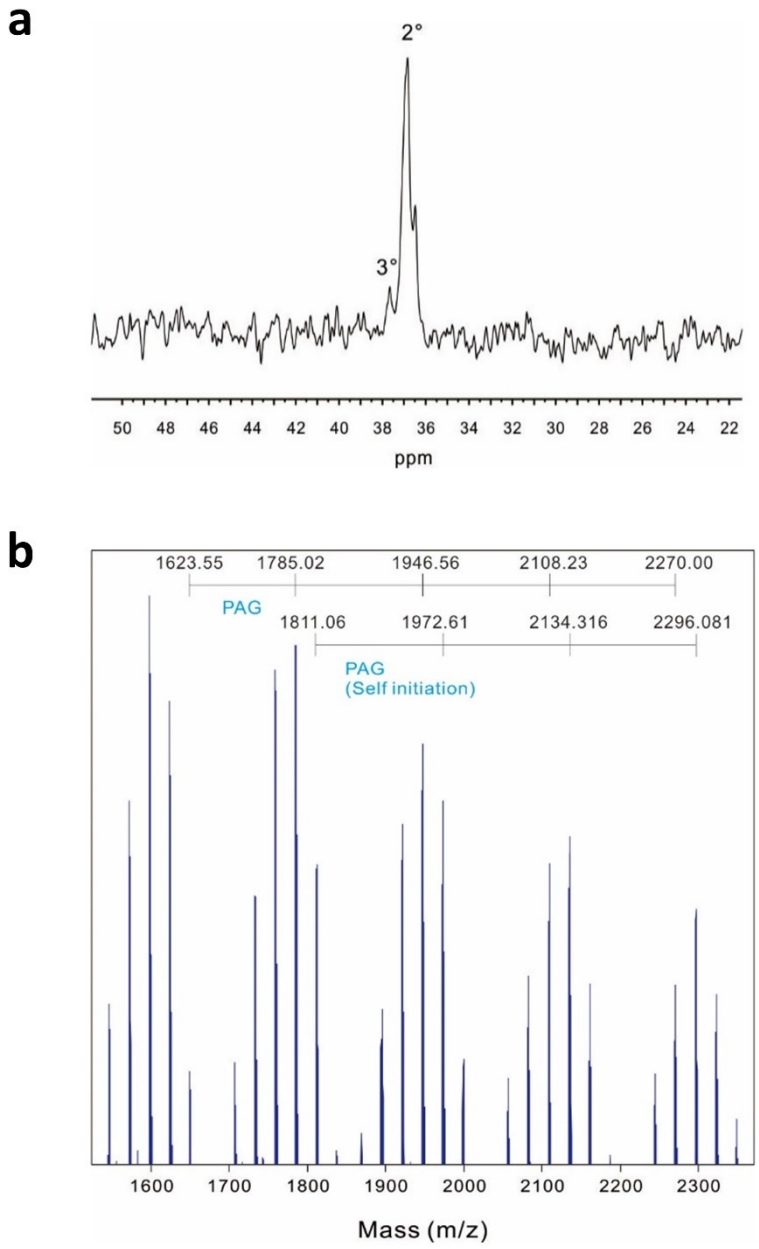


Figure 5. (a) Detailed ^{15}N-NMR spectrum of PAG_{66} (polymer 5) in DMSO-d_6. (b) Expanded MALDI-ToF mass spectrum of the PAG_{13} (polymer 1) from 1600 to 2300 Da. The spacing of the signals corresponds to the mass of the respective monomers (AG: 161.2 g/mol).

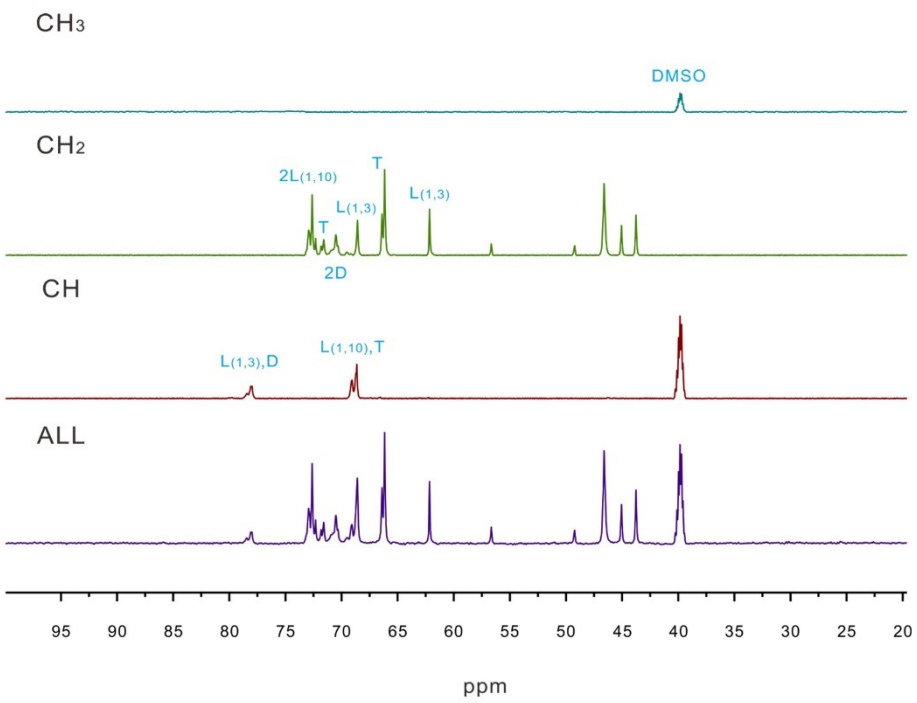
counterion (TMP (134.17) + monomer of PAG (161.2) × 11 + K^+ (39.1)). However, the peaks corresponding to polymers of self-initiated PAG was also observed. During the polymerization with potassium methoxide, a fraction of the monomer acts as an initiator and the polymer formed as a side reaction may have a cyclic form, albeit the use of the slow monomer addition to keep the concentration of the monomers low during the reaction.

Furthermore, the hyperbranched nature of the PAG polymers was assessed by analyzing the degree of branching (DB) using a detailed analysis of the ^{13}C NMR spectra (Figure 6, Figure 7a) based on a previously reported equation.[27] The resulting DB indicated the percentage of the branched segment within the PAG polymer chains. The DB of the selected polymer $PBAG_{66}$ was determined to be approximately 0.41, which was slightly lower than the conventional hyperbranched polymers (0.4–0.6). We postulated that the longer spacer unit in the BAG monomer limited the branching of terminal hydroxyl group compared to a glycidol monomer.

3.2 Biocompatibility assay

We measured the cytotoxicity of PAGs, such as PAG_{17} (Polymer 2) and PAG_{50} (Polymer 4) to confirm their potential in biomedical application. Each polymer was tested in murine macrophage cell line condition. RAW264.7 are used as a model normal cell line. The cell viability of the polymers was measured using WST-1 assay, which are generally used for in vitro cytotoxicity assays of polymers and nanomaterials. Unlike the commonly used cell viability method MTT assay, which requires a solubilizing process, the WST-1 assays have the high sensitivity and wide measurement range. As described in Figure 7b, the cell viability of each cell line treated with diverse concentrations of PAG_{17} was higher than 90% up to a concentration of 500 μg/mL. In the case of PAG_{50}, which has more content of amine moieties, the study showed a considerable toxicity due to its toxic free amine groups; Therefore, the cell viability decreased dramatically up to a concentration of 250 μg/mL. Albeit many polyamines are reported to display considerable cytotoxicity due to their free amine groups associated with tight cell binding,[28,29] our PAG polymers showed remarkably lower cellular toxicity; this is due to the amine groups being sheathed by the polyglycerol shell, producing optimum cell viability.

a



b

Region	Chemical shift(PPM)	Relative Integral Values
		PAG_{66}
$L_{(1,10)}$	72.41~73.48	1.33
Terminal	71.31~72.11	0.81
Dendritic	70.06~71.21	0.82
$L_{(1,3)}$	61.96~62.47	1.00

Figure 6. DEPT spectra of PAG_{66} polymer (polymer 5) in DMSO-d_6 (b) Calculation of degree of branching based on the ^{13}C NMR spectra of PAG_{66} polymer (polymer 5).

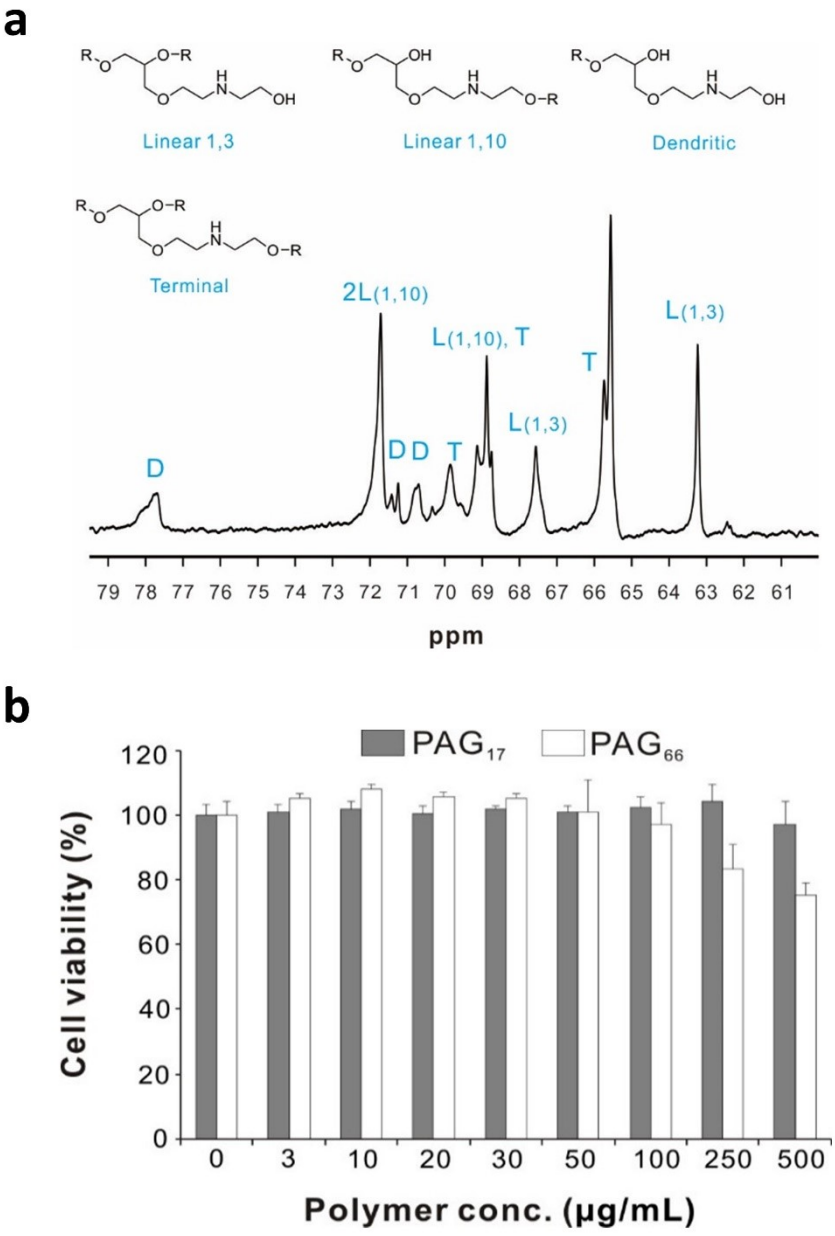


Figure 7. (a) Detailed ^{13}C NMR spectrum of PAG_{66} polymer (polymer 5) in D_2O with assignment of respective linear, dendritic, and terminal groups within the structure. (b) In vitro cell viability assay of polymers. (Gray) PAG_{17} (polymer 2) and (white) PAG_{50} (polymer 4) determined by WST-1 assays using RAW264.7 cell lines.

3.3 Singlet oxygen generation assay

Reactive oxygen species (ROS) are exceptionally reactive chemical species containing reactive oxygen. Examples of ROS are peroxides, superoxide, hydroxyl radical and singlet oxygen. Reactive oxygen species are essential component of our body that affect cell signaling and homeostasis. Among the various ROS, singlet oxygen can be easily generated by energy transfer or charge transfer. Since singlet oxygen is produced by energy transfer, it is widely used in photodynamic therapy.

We hypothesized that lone pair electron of amine functional group of polyamine react with light to generate singlet oxygen. We suggested singlet oxygen generation mechanism in Figure 8. Lone pair electron of amine group of PAG react with light to generate free electron. Free electron generated react with triplet oxygen to generate superoxide radical. The superoxide radical generated react with lone pair electron of positively charged PAG to produce singlet oxygen. To identify the hypothesis, We performed the 9,10-anthracenediyl-bi(methylene)dimalonic acid (ABDA) assay. The ABDA assay is common experiment method to identify singlet oxygen generation. ABDA is easily degraded when it reacts with singlet oxygen. Therefore, the generation of singlet oxygen can be confirmed by examining the reduction in the amount of ABDA when photo irradiation is applied. First of all, We lighted solution containing ABDA and PAG. ABDA is degraded when photo irradiation is applied to the solution containing ABDA and PAG. Therefore, we assumed that PAG generates singlet oxygen when photo irradiation is applied (Figure 9g,h). However, ABDA is not degraded at pH 10 (Figure 9i). This is because the amine groups of the PAG are not charged when the pH is high. A PAG with minimalized charge will inevitably lose aqueous solubility. When light irradiation was applied, a solution containing only-ABDA was not degraded (Figure 9a-c). When photo irradiation was applied to the mixed solution of PG and ABDA, the amounts of ABDA have not changed. (Figure 9d-f). These results confirm that the PG backbone structure does not affect the degradation of ABDA. However, another polyamine, PEI, was observe to degrade ABDA (Figure 9j-k). PEI also exhibits low degradation at pH 10 due to the solubility problem (Figure 9i). Considering all the results, we assumed that the amine groups of the polymer interact with light to generate singlet oxygen.

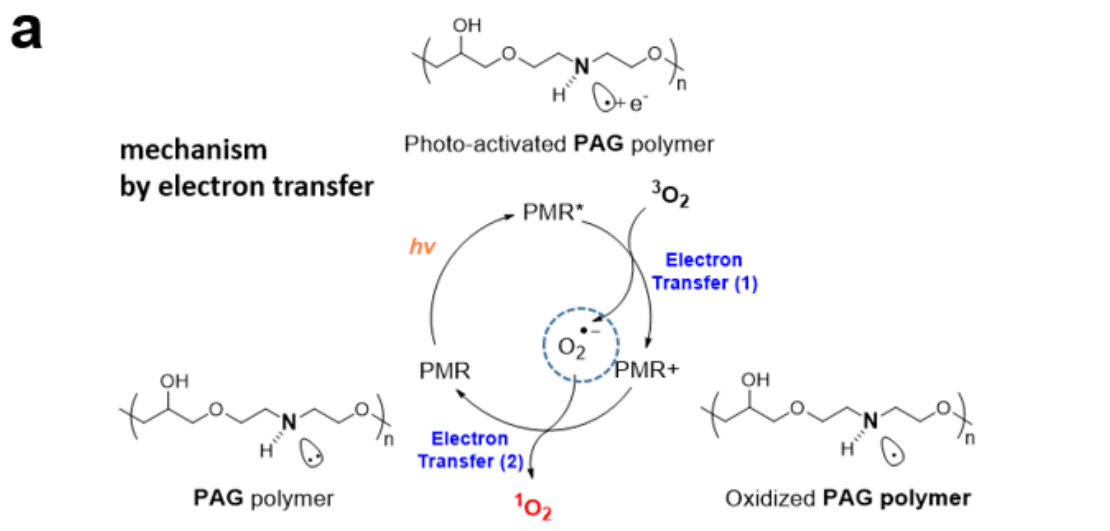


Figure 8. (a) Potential singlet oxygen generation mechanism. (b) Molecular orbital diagrams for triplet oxygen, superoxide radical and singlet oxygen.

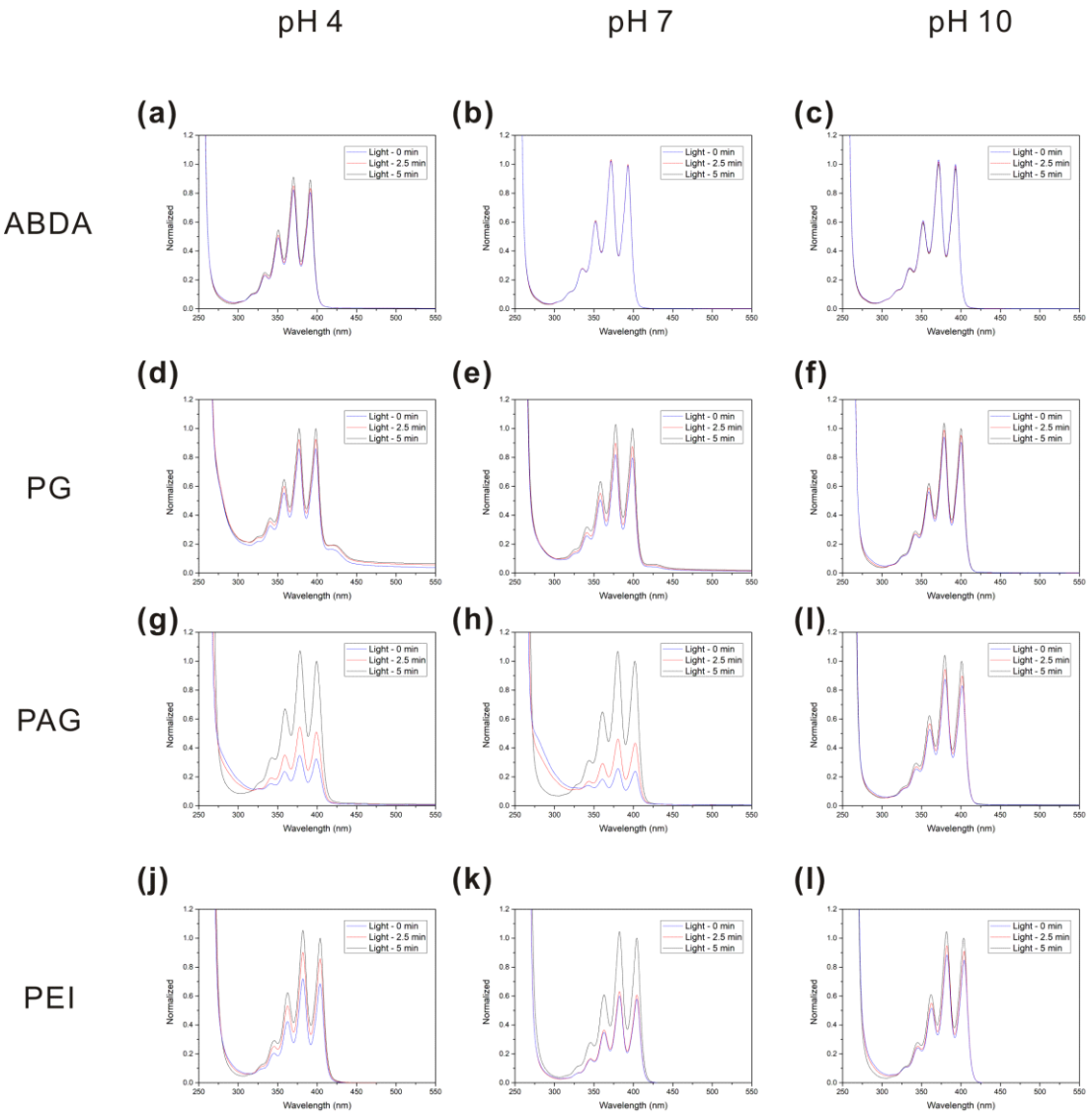


Figure 9. Absorbance decrease of 9,10-anthracenediyl-bi(methylene)dimalonic acid (ABDA) by singlet oxygen ($^1O_2$) production. Conditions: [PAG polymer] = 10 μM; [ABDA] = 100 μM; irradiation with 40% intensity of 1 sun light. Absorbance was obtained every 2.5 min.

4. Conclusion

In brief, we report a one-pot synthesis of hyperbranched polyamines. A novel BAG monomer was designed and polymerized using anionic ring-opening multi-branching polymerization to synthesize a well-controlled PBAG polymer. Following deprotection of PBAG yielded the hyperbranched polyamino glycerols (PAG). The polymerization was successfully analyzed by ^{1}H, ^{13}C and ^{15}N-NMR, GPC, MALDI-ToF, and DSC measurements. The high biocompatibility of PAG certainly demonstrate its powerful potential for use in biochemical and pharmaceutical applications. Furthermore, we have confirmed that PAG generates singlet oxygen. Therefore, PAG can be used for photodynamic therapy. We expect that the new class of functional epoxide monomer and polymers developed in this study will contribute to the advancement of polyglycerol-based polymers and will be bright candidates for emerging materials and biomedical applications.

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